The Recombinant Murine Leukemia Virus Reverse Transcriptases, The Genes Encoding and The Method For Expressing It

Technology Category

This invention involves recombinant reverse transcriptases, their coding genes and expression methods in the field of biotechnology. This invention particularly involves recombinant murine leukemia virus reverse transcriptases, their coding genes and expression methods.

Background of the Invention

Reverse transcriptase (RT) is a kind of DNA polymerase encoded by retroviruses, which can synthesize DNA using DNA or RNA as template. Reverse transcriptase, which can convert RNA into cDNA, is widely used in molecular biology, including constructing cDNA libraries and analyzing the amount of RNA in biological samples by RT-PCR. Nowadays the dominant RT in the market is murine leukemia virus reverse transcriptase (MLV-RT).

MLV-RT is composed of two functional domains, a DNA polymerase domain at the N-terminus and a ribonuclease (RNase) H domain at the C-terminus. These two domains can be expressed separately without affecting the function of each other. The first generation of recombinant MLV-RT used in cDNA synthesis contained only the DNA polymerase domain, with the RNase H domain deleted. Though the enzymatic activity is similar to that of full-length MLV-RT, its processivity is relatively poor, resulting in short cDNAs. It was found that although the RNase H domain does not affect the DNA polymerase activity of MLV-RT, it affects the enzyme's processivity. The reason is that the RNase H domain binds to the template/primer complex, and thereby increases the enzyme's affinity for the template/primer. When the 524th Asp, a key residue in the RNase H active site, is replaced with Asn by site-directed mutagensis, the resulting mutant enzyme, MLV-RT-D524N, does not demonstrate any RNase H activity, but retains the DNA polymerase activity and high affinity for the template/primer. This mutant enzyme, which is patented by Invitrogen under trade mark Superscript II, is currently widely used. However, this mutant enzyme is still not perfect; its processivity is still not ideal and the most common problem encountered is the formation of short, less than full-length products.

Summary of the Invention

This invention provides murine leukemia virus reverse transcriptases, named MLV-RT-Q84X, wherein the 84th amino acid residue (Q84) from the N-terminus is replaced with X, which represents a residue with a side chain shorter than that of glutamine.

The invention further provides murine leukemia virus reverse transcriptases, named MLV-RT-Q84X-D524N, wherein the 524th aspartic acid residue from the N-terminus is replaced with asparagines (Asn), and the 84th amino acid residue (Q84) from the N-terminus is replaced with X, which represents a residue with a side chain shorter than that of glutamine.

The invention further provides murine leukaemia virus reverse transcriptases, MLV-RT-Q84X and MLV-RT-Q84X-D524N, wherein X is preferably chosen from alanine (Ala), serine (Ser), asparagine (Asn), or aspartic acid (Asp). Alanine is especially favoured.

The invention further provides the coding sequences of the recombinant murine leukemia virus reverse transcriptases.

The invention further provides a method of expressing the recombinant murine leukemia virus reverse transcriptases comprising: a) transforming the murine leukaemia virus reverse transcriptases expressing plasmids into *Escherichia coli*; b) culturing the clones to express recombinant murine leukemia virus reverse transcriptases. These recombinant murine leukemia virus reverse transcriptases are a series of MLV-RT proteins wherein the 84th amino acid residue (Q84) from N-terminus is replaced by a residue that has a side chain shorter than that of glutamine.

The invention also provides a method of expressing the recombinant murine leukemia virus reverse transcriptases comprising: a) transforming the murine leukaemia virus reverse transcriptases expressing plasmids into *Escherichia coli*; b) culturing the clones to express recombinant murine leukemia virus reverse transcriptases. These recombinant murine leukemia virus reverse transcriptases are a series of MLV-RT proteins wherein the 524th aspartic acid residue from the N-terminus is replaced with asparagine, and the 84th amino acid

residue (Q84) from N-terminus is replaced by a residue that has a side chain shorter than that of glutamine. The 84th amino acid is preferably replaced by alalnine.

The invention further provides nucleotide sequences of plasmids pTacRT-Q84N-D524N and TacRT-Q84A-D524N as described in SEQ1 and SEQ 3, respectively. pTacRT-Q84N-D524N and TacRT-Q84A-D524N express murine leukaemia virus reverse transcriptases with amino acid sequences described in SEQ2 and SEQ4, respectively. The host cell expressing these proteins is *Escherichia coli* BL21. Both SEQ 1 and SEQ 3 are composed of 7488 nucleotides with an open reading frame between the 1515th and 3527th nucleotides. Both SEQ2 and SEQ4 are composed of 671 amino acids.

Brief Description of the Figures

FIGURE1 SDS-PAGE analysis of purified MLV-RT-Q84A-D524N

FIGURE2 Kinetic analysis of MLV-RT-Q84A-D524N and MLV-RT-D524N

FIGURE3 The first strand cDNA synthesis by MLV-RT-Q84A-D524N and MLV-RT-D524N .

FIGURE4 SDS-PAGE analysis of purified MLV-RT-Q84N-D524N

FIGURE5 DNA polymerase activity assay of MLV-RT-Q84N-D524N

Experimental Details

Experiment 1: Preparation of MLV-RT-Q84A-D524N

1, Construction of plasmid pTacRT-Q84A-D524N

The Q84A substitution was introduced into MLV-RT-D524N to generate MLV-RT-Q84A-D524N.

The Q84A mutation in the MLV-RT-D524N（*Blain, S.W. & Goff, S.P. (1995) J. Virol. 69, 4440-4452.*）backbone was constructed by replacing the AflII-MfeI fragment of pTacRT-D524N (nt1467-2058) with two PCR-derived fragments AflII-EcoRI and EcoRI-MfeI . The 300bp AflII-EcoRI fragment was generated using forward primer （5'GTGGAATTGTGAGCCGA） and a mutation specific reverse primer Q84A-AP (5'CGGAATTCCC**GCG**TCCAACAGTCTCTGTA) bearing silent mutations creating a restriction site; the 300bp EcoRI-MfeI fragment was generated using reverse primer

（5'TGGGAGTCTGGTCCAGG） and a mutation specific forward primer Q84A-SP (5'CGGAATTCTGGTACCCTGCCAGTC) bearing silent mutations creating the same restriction site as created in the 5' fragment. The codon for alanine was built in the mutation specific primers. The restriction sites built in the primers are underlined. The AflII-EcoRI fragment (nt1467-1770) and EcoRI-MfeI fragment (nt1770-2058) were inserted into a 6.9 kb vector, pTacRT-D524N, which was digested with AflII/-MfeI. The ligation mixture was transformed into *Escherichia coli* Top10 and pTacRT-Q84A-D524N clones were picked based on restriction enzyme deigestion analysis. The result of nucleotide sequencing showed that the sequence of pTacRT-Q84A-D524N was the same as the sequence in SEQ 1.

2. Expression of recombinant reverse transcriptases in *Escherichia coli*

Escherichia coli BL21 cells transformed with pTacRT-Q84A-D524N were inoculated in LB medium containing 100μg/ml ampicillin at 37°C. When the cells were grown to a density of OD_{600} 0.5, IPTG was added to the medium at the final concentration of 0.5 mM to induce RT expression. The cells were cultured for additional 2-3 hours at 37 °C. At the end of the induction, the cells were harvested by centrifugation and washed once with 50 mM ice-cold Tris-HCl (pH 7.5) for further RT purification.

3, Purification of the RT proteins

The cells were resuspended in buffer A (20mM sodium phosphate (pH 7.4), 0.5 M NaCl) containing lysozyme at a final concentration of 0.5mg/ml and incubated on ice for 30 min. The suspension was briefly sonicated and then cleared of debris by centrifugation. Afte HiTrap chelating HP column (Pharmacia) purification, the purity of RT was higher than 80%, as analyzed by SDS-PAGE. The enzyme was purified to near homogeneity by MonoS (Pharmacia) fast protein liquid chromatography (FPLC), as analyzed by SDS-PAGE. MLV-RT-Q84A-D524N is a nearly homogenous band of 76 kD in the gel after Coomasia Brilliant Blue staining (Figure1). Figure1: M, Molecular Weight Standard; 1, 5 μg protein; 2, 2 μg protein.

4. Homopolymer assays and Kinetic analysis

Typical assays were performed at 37°C using 10 ng of RT in 50μl of reaction containing 60 mM Tris.HCl (pH 8.0), 75 mM NaCl, 0.7 mM $MnCl_2$, 5 mM DTT, 12 μg/ml poly(rA)

template, 6 μg/ml oligo(dT)$_{18}$ primer, 10 μCi/ml ^{32}P-labeled dTTP (1Ci=37GBq) and 12 μM unlabeled dTTP. At each time point, 4 μl of the reaction was removed and spotted on DE81 paper (Whatman). The paper was washed twice with 2 X standard saline citrate (SSC), followed by scintillation counting.

To measure the kinetic parameters, the enzyme was added to the reaction mix to initiate the reaction. The radioactivity retained on the paper, in comparison with the total radioactivity in each sample, was used to determine the amount of dTTP incorporated into the product. The kinetic parameters were determined by double reciprocal plot (Figure 2). While MLV-RT-Q84A-D524N and MLV-RT-D524N displayed comparable affinities (*Km*) for dTTP (11.04 μ M and 12.94 μ M, respectively), the catalytic activity (*Vmax*) for dTTP of MLV-RT-Q84A-D524N was 3.2 times the level of MLV-RT-D524N (0.41 μmol.min-1.ng-1 and 0.13 μmol.min-1.ng-1, respectively) (Table.1).

Table 1. Comparason of Kinetic Patameters between MLV-RT-Q84A-D524N and MLV-RT-D524N

Enzyme	Vmax ($\mu mol.min^{-1}.ng^{-1}$)	Km (μM)
RT-D524N	0.13±0.04	12.94±2.08
RT-Q84A-D524N	0.41±0.04	11.05±0.72

5, First strand cDNA synthesis:

Here we show the difference in first strand cDNA synthesis between MLV-RT-Q84A-D524N and MLV-RT-D524N.

The total RNA of Rat-2 cells was isolated using the RNeasy kit (Qiagen) following the manufacturer's instructions. The first strand cDNA synthesis was performed in 20 μl of reaction containing 50 mM Tris.HCl (pH 8.0), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 500 μM dNTPs, 20u RNasin, 10 μCi α-^{32}P-dCTP (1Ci=37 GBq) , 1 μg of total RNA template. The reaction mixture was preheated at 42°C for 2min before 0.5 μg RT was added to initiate DNA synthesis. The reaction was carried out at 42°C for 1 hour and then stopped by heating at 70°C for 15min. The products were analyzed by electrophoresis on a 1.4% alkaline agarose gel, followed by autoradiography. As shown in Figure 3, RT-Q84A-D524N synthesizes longer and more cDNAs than RT-D524N.

Example 2: Preparation of MLV-RT-Q84N-D524N

1, Construction of plasmid pTacRT-Q84N-D524N

The of Q84N substitution was introduced into MLV-RT-D524N to generate MLV-RT-Q84N-D524N.

The Q84N mutation in the MLV-RT-D524N (*Blain, S.W. & Goff, S.P. (1995) J. Virol. 69, 4440-4452*) backbone was constructed by replacing the AflII-MfeI fragment of pTacRT-D524N (nt1467-2058) with two PCR-derived fragments AflII-EcoRI and EcoRI-MfeI . The 300bp AflII-EcoRI fragmentwas generated using forward primer (5'GTGGAATTGTGAGCCGA) and a mutation specific reverse primer Q84N-AP (5'CGGGATCCC**GTT**GTCCAACAGTCTCTGTA) bearing silent mutations creating a restriction site; the 300bp EcoRI-MfeI fragment was generated using reverse primer (5'TGGGAGTCTGGTCCAGG) and a mutation specific forward primer Q84N-SP (5'CGGGATCCTGGTACCCTGCCAGTC) bearing silent mutations creating the same restriction site as created in the 5' fragment. The codon for asparagine was built in the mutation specific primers. The restriction sites built in the primers are underlined. The AflII-BamHI fragment (nt1467-1770) and BamHI -MfeI fragment (nt1770-2058) were inserted into a 6.9 kb vector, pTacRT-D524N, which was digested with AflII and MfeI. The result of nucleotide sequencing showed that the sequence of pTacRT-Q84N-D524N was the same as the sequence in SEQ 3.

2, Expression of recombinant reverse transcriptases in *E.coli*

Escherichia coli BL21 cells transformed with pTacRT-Q84N-D524N were inoculated in LB medium containing 100μg/ml ampicillin at 37°C. When the cells were grown to a density of OD_{600} 0.5, IPTG was added to the medium at the final concentration of 0.5 mM to induce RT expression. The cells were cultured for additional 2-3 hours at 37 °C. At the end of the induction, the cells were harvested by centrifugation and washed once with 50 mM ice-cold Tris-HCl (pH 7.5) for further RT purification.

3, Purification of recombinant reverse transcriptase

The cells were resuspended in buffer A (20mM sodium phosphate (pH 7.4), 0.5 M NaCl) containing lysozyme at a final concentration of 0.5mg/ml and incubated on ice for 30 min. The suspension was briefly sonicated and then cleared of debris by centrifugation. After HiTrap chelating HP column (Pharmacia) purification, the purity of RT was higher than 80%,

as analyzed by SDS-PAGE. The enzyme was purified to near homogeneity by MonoS (Pharmacia) fast protein liquid chromatography (FPLC), as analyzed by SDS-PAGE. MLV-RT-Q84N-D524N is a nearly homogenous band of 76 kD in the gel after Coomasia Brilliant Blue staining (Figure 4). Figure 4: M, Molecular Weight Standard; 1, 1 μg protein; 2, 2 μg protein; 3, 5 μg protein.

5, Homopolymer Substrate Assays

Typical assays were performed at 37°C using 10 ng of RT in 50μl of reaction containing 60 mM Tris.HCl (pH 8.0), 75 mM NaCl, 0.7 mM $MnCl_2$, 5 mM DTT, 12 μg/ml poly(rA) template, 6 μg/ml oligo$(dT)_{18}$ primer, 10 μCi/ml ^{32}P-labeled dTTP (1Ci=37GBq) and 12 μM unlabeled dTTP. At each time point, 4 μl of the reaction was removed and spotted on DE81 paper (Whatman). The paper was washed twice with 2 X standard saline citrate (SSC), followed by autoradiography.

MLV-RT-Q84N-D524N manifested higher activities than RT-WT-H but had a similar activity to MLV-RT-Q84A-D524N (Figure 5).

Application

Based on the high-resolution structure of a catalytically active fragment of MLV-RT, we discovered that the 84th amino acid residue of this enzyme, glutamine (Q84), which is located in the active site, regulates the catalytic activity of the enzyme. The long side chain of this residue presumably blocks the elongation of the products and affects the DNA polymerase activity and processivity. By replacing Q84 with an amino acid residue with a side chain shorter than that of glutamine, we generated a new enzyme, MLV-RT-Q84X, wherein X is an amino acid with a side chain shorter than that of glutamine, like alanine (Ala), serine (Ser), asparagine (Asn), or aspartic acid (Asp) and so on. In this invention the Q84X substitution was also introduced into MLV-RT-D524N, togenerate MLV-RT-Q84X-D524N. These recombinant murine leukemia virus reverse transcriptases demonstrate higher enzyme activity and processivity than MLV-RT-D524N (Superscript II). These new enzymes are expected to be widely used in the field of biotechnology, particularly for cDNA synthesis.